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Short communication

Contribution of herbal principles towards cytoprotective, antioxidant and anti-*Rhizopus* activities

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Abstract

Herbal extracts (twenty extracts) obtained from 20 medicinal and aromatic plants were evaluated for their cytoprotectivity and antioxidant properties. Total phenol content and anti-*Rhizopus* activity were also determined. Results indicated that, out of 20 extracts evaluated, radical scavenging capacity and anti-*Rhizopus* activity were observed in aqueous extract of *Ocimum tenuiflorum*, *Leucas aspera*, *Terminalia arjuna*, *Glycyrrhiza glabra* and *Nyctanthes arbortristis* in a dose dependent manner. The total phenolic content was observed to be 1289, 3837, 372, 2831 and 1892 μg GAE/g for *O. tenuiflorum*, *L. aspera*, *T. arjuna*, *G. glabra* and *N. arbortristis* respectively. The antioxidant activity correlates with the phenolic content of the extracts. At 1 mg/ml the above extracts showed 98% protection on erythrocyte and buccal cell oxidation. In DNA oxidation studies, higher protection was observed in *O. tenuiflorum*, *L. aspera* and *N. arbortristis* extracts. These results demonstrate that the cytoprotectivity and antioxidant potency of these extracts could be the basis for their ayurvedic health promoting potential. They could serve as new sources of natural antioxidants or nutraceuticals with potential applications in reducing oxidative stress conditions.

Keywords: Antioxidant; Anti-*Rhizopus*; Cytoprotective; DPPH; Herbal extracts; Oxidation

1. Introduction

Many studies have established relation between oxidative stress, cellular senescence and some diseases (Meghashri et al., 2010). In physiological and pathological pathways, reactive oxygen species can cause DNA mutation, protein oxidation and lipid peroxidation, contributing to the development of atherosclerosis, inflammation, neurodegenerative diseases, cataracts, cancer and aging (Esra and Salih, 2012). Free radicals are generated during normal cellular metabolism, and their effect is neutralized by antioxidant molecules present in the body. However, the balance between the oxidants and antioxidant molecules is disturbed by excess free radicals derived from exogenous sources like ozone, exposure to UV radiations and cigarette smoke (Roselli et al., 2011). A potent scavenger or quencher of these free radical species may serve as a possible preventive measure for free radical mediated diseases. There is growing concern over synthetic additives, such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT), which have long been used to preserve food from oxidation (Imaida et al., 1983; Wong and Kitts, 2006). Therefore, there is interest in natural products and plant extracts as possible sources of free radical scavengers in form of food additives.

Fruits and vegetables get infected by pathogen during harvesting and packing. *Rhizopus stolonifer* is reported to cause food spoilage and decay in fruits, particularly peaches, strawberries, raspberries and grapes usually after harvest (Northover and Zhou, 2002). The post-harvest losses derived from *Rhizopus* rot are rapidly increased by the spread of the fungus to the adjacent fruits during ripening because the pathogen is not efficiently controlled by registered fungicides and treatments. In an attempt to reduce the use of chemicals as preservatives, due to the concern about human health and environmental pollution,

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new approaches are being developed such as the use of medicinal and aromatic plant extracts with antimicrobial and antifungal activities.

In the present investigation, the protective effect of herbal extracts on free radical induced damage on erythrocytes, buccal cells and DNA were assessed along with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability. The total phenol content and anti-*Rhizopus* potential were also determined.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), diphenylboric acid-2-amino ethyl ester, ascorbic acid, and butylhydroxyanisole (BHA) were purchased from HiMedia Laboratories Pvt Ltd., Mumbai, India. Vanillin was purchased from Merck, India. Lambda phage DNA was procured from Bangalore Geni, Bangalore, India. All other chemicals and solvents were of analytical grade and purchased from common sources.

2.2. Plant materials and extraction

The plants were collected in the Northern and Southern provinces in India. Plant materials (Table 1) were authenticated at the Department of Studies in Botany, University of Mysore, Mysore. Each plant material was air dried in the dark and ground to powder thereafter. Five grams each of different parts of the plants was successively macerated at 4 °C for 24 h with 200 ml of water until exhaustion of the material. The aqueous extracts were separately lyophilized. Dry extracts were stored in glass vials at 4 °C until tested and analyzed.

Table 1
DPPH radical scavenging potential of herbal extracts.

Sample	Taxon	Family	Organ	IC_{50} (μg/ml)
1	*Ocimum tenuiflorum*	Lamiaceae	Leaf	13.74±1.43
2	*Coleus forskohlii*	Lamiaceae	Leaf	58.44±4.67
3	*Asparagus racemosus*	Asparagaceae	Root	56.11±3.76
4	*Centella asiatica*	Mackinlayaceae	Leaf	41.13±1.39
5	*Andrographis paniculata*	Acanthaceae	Leaf	37.30±3.81
6	*Ocimum basilicum*	Lamiaceae	Leaf	27.11±1.30
7	*Ocimum gratissimum*	Lamiaceae	Leaf	37.82±1.38
8	*Coleus blumei*	Lamiaceae	Leaf	38.17±3.81
9	*Coleus forskolii*	Lamiaceae	Leaf	158.58±1.36
10	*Leucas aspera*	Lamiaceae	Leaf	12.13±0.98
11	*Terminalia arjuna*	Combretaceae	Bark	19.04±0.70
12	*Acorus calamus*	Acoraceae	Root	57.01±1.40
13	*Glycyrrhiza glabra*	Fabaceae	Root	24.90±2.80
14	*Nyctanthes arbortristis*	Oleaceae	Leaf	14.14±0.38
15	*Emblica officinalis*	Euphorbiaceae	Fruit	616.41±0.92
16	*Terminalia bellerica*	Combretaceae	Fruit	63.13±2.27
17	*Terminalia chebula*	Combretaceae	Fruit	83.12±1.11
18	*Withania somnifera*	Solanaceae	Root	120.09±2.56
19	*Evolvulus alsinoides*	Convolvulaceae	Leaf	132±0.81
20	*Tridox procumbens*	Asteraceae	Leaf	89±0.89
21	BHA			17.55±1.96
22	Ascorbic acid			7.36±0.36

2.3. Determination of total phenolic content

The total phenolic content of each of the herbal extracts was determined colorimetrically using the Folin–Ciocalteu method (Meghashri et al., 2010). A sample aliquot of 100 μl was added to 900 μl of water, 5 ml of 0.2 N Folin–Ciocalteu reagent and 4 ml of saturated sodium carbonate solution (100 g/l). The absorbance was measured at 765 nm after incubation for 2 h at room temperature. The total phenolic content was expressed as gallic acid equivalent (GAE) in micrograms per gram sample.

2.4. Phytochemical screening by TLC combined with DPPH staining

In order to evaluate antioxidant activity, a method based on the reduction of DPPH was carried out (Blois, 1958). Two hundred micrograms of each crude extract was spotted on silica-gel $60F_{254}$ plates (Merck, India) and developed in suitable solvent system: EtOAc/MeOH/H_2O (60:20:5; aqueous extracts). Three plates were prepared under the same conditions for each extract, one for the antiradical test and the others for the detection of the nature of the compounds using plant drug reagents (Wagner and Bladt, 1996). The first plate was sprayed with a methanolic solution of DPPH (2 mg/ml). Twenty micrograms each of ascorbic acid and BHA were used as positive controls, considering antiradical activity as yellow spots on purple background. The second plate was sprayed with vanillin/H_2SO_4 reagent heated at 110 °C for 5 min to detect the presence of polyphenolic and other groups of compounds (orange-yellow spots indicate polyphenolic compounds). The third plate was sprayed with Natural Products-PEG reagent and observed at UV-365 nm (flavonoids are detected as yellow-orange fluorescent spots).

2.5. Cytoprotective studies of herbal extracts on erythrocyte and buccal cells

Erythrocytes were obtained from healthy consenting donors. Heparinized blood was centrifuged at 1000 *g* for 15 min. After removal of plasma and buffy coat, the erythrocytes were washed thrice with PBS (20 mM, pH 7.4, NaCl—0.9%) at room temperature and resuspended in PBS four times its volume for subsequent analysis (Suwalsky et al., 2007). The buccal cells were scraped from the cheek and washed thrice with PBS, centrifuged and resuspended in PBS. Erythrocytes and buccal cells were incubated with aqueous extracts (1 mg/ml) for 5 min and then hydrogen peroxide (30 mM); ferric chloride (80 μM) and ascorbic acid (50 μM) were added and incubated at 37 °C for 1 h. The reaction mixture was gently shaken while being incubated. After incubation, 50 μl of acridine orange and ethidium bromide dye were added to buccal cells and observed under fluorescent microscope at 40×. The morphology of erythrocytes was observed in a optical microscope.

2.6. DPPH radical scavenging assay

The effect of different herbal extracts on DPPH radical was estimated according to Meghashri et al. (2010). Herbal extracts

(0–150 μg/ml) in 200 μl aliquot was mixed with 100 mM Tris–HCl buffer (800 μl, pH 7.4), then added to 1 ml of 500 μM DPPH in ethanol (final concentration of 250 μM) and left to stand for 20 min at room temperature. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capacity to scavenge DPPH radical was calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100$$

where A = absorbance.

2.7. DNA protection assay

DNA protection ability of herbal extracts was performed using lambda phage DNA (Meghashri et al., 2010). Briefly, λ phage DNA (0.6 μg) was subjected to oxidation using Fenton's reagent (0.3 mM hydrogen peroxide, 0.5 μM ascorbic acid and 0.8 μM ferric chloride) in presence and absence of herbal extracts (0.8 mg/16 μl) for 2 h at 37 °C. The samples were subjected to electrophoresis on 1% agarose for 2 h at 50 V DC. Gels were stained with ethidium bromide (0.5 μg/ml) and documented (Herolab, Germany).

2.8. Evaluation of antifungal activity

The fungus used in the assay was *R. stolonifer*, and it was acquired from Microbial Type Culture Collection (MTCC 2189). *R. stolonifer* was grown on potato-dextrose agar (PDA) medium on Petri plates. The antifungal activity was evaluated by the method of mycelial growth (Zhang et al., 2006). Plant extracts were resuspended in sterile Eppendorf with DMSO and were added to sterile PDA medium. All extracts were assayed in a final concentration of 250 ppm. Benzoic acid and BHA were used as positive controls at the same concentration. Five millimeters of *R. stolonifer* mycelium (taken from a 5-day-old fungi culture) were inoculated in fresh medium containing DMSO (control) and in fresh medium containing DMSO plus plant extract. After incubation for 2 h at 25 °C in darkness, growth zones were measured and converted into percentage of inhibition: [(Control − Treated)/Control) × 100].

2.9. Statistical analysis

All experiments were carried out in triplicates, and data were shown as mean ± standard deviation (SD). SPSS 10.0.5 version for Windows (SPSS Software Inc., USA) computer program was used for statistical analysis. The significance of the study was assessed by one-way ANOVA, followed by post hoc comparison test. Correlations between quantitative properties were evaluated by calculating the Duncan and Dunnett's coefficient. Statistical significance value was set at $p < 0.05$.

3. Results and discussion

In this study, the following were investigated in the selected plant extracts: total phenolic content, cytoprotection of human erythrocytes and buccal cells, protection to DNA oxidation, DPPH radical scavenging activity and anti-*Rhizopus* activity. The presence of phenolic compounds in herbal extracts has been previously reported (Meghashri et al., 2010; Roselli et al., 2011). High total phenolic content was observed in aqueous extract *Ocimum tenuiflorum*, *Leucas aspera*, *Terminalia arjuna*, *Glycyrrhiza glabra* and *Nyctanthes arbortristis* (1289, 3837, 372, 2831 and 1892 μg GAE/g respectively) when compared to other plant extracts.

In the DPPH free radical scavenging capacity assay by TLC, the spots in the extracts on the TLC that produced yellow color on purple background were considered as antioxidants. TLC profiles of the extracts obtained from *O. tenuiflorum*, *L. aspera*, *T. arjuna*, *G. glabra* and *N. arbortristis* showed the presence of flavonoids (data not shown) and phenolic compounds with R_f value 0.73 as well as positive result for DPPH staining compared to other extracts (Fig. 1). Therefore, it was deduced that the activity was due to polyphenolic compounds.

The results of cytoprotectivity on erythrocytes and buccal cells on oxidation as indicated in the optical micrographs (Fig. 2a) showed the protective ability of aqueous extracts of *O. tenuiflorum*, *L. aspera*, *T. arjuna*, *G. glabra* and *N. arbortristis* on erythrocyte membrane protection when compared to other plant extracts. Erythrocytes treated with hydrogen peroxide showed the appearance of echinocytes, indicating damage to the cell membrane. While in plant extracts treated samples, the presence of normal cells can be seen in addition to oxidized cells indicating the protective role of these herbal extracts. The fluorescent micrograph (Fig. 2b) showed the protective ability of aqueous extracts on buccal cells. Cells treated with hydrogen peroxide showed the appearance of clustering and cell disruption, while in plant extracts treated samples, normal cells can be seen indicating protectivity. Cytoprotective ability of these plant extract may be attributed to their polyphenolic components. There are no reports of cytoprotective abilities of polyphenols in these herbal extracts. However, reports are available in the literature from other plant sources (Lima et al., 2007). Erythrocytes have been extensively used to study oxidative stress, which represent a simple cell model. Oxidants

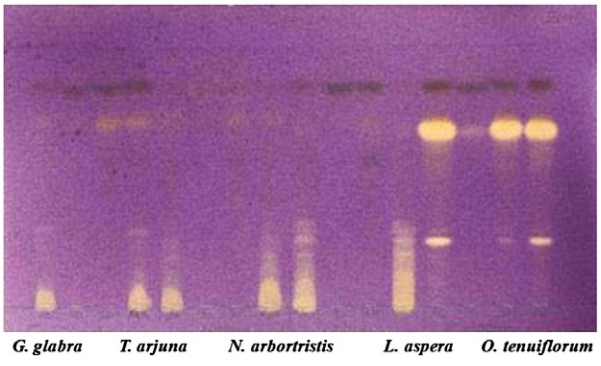

Fig. 1. Chromatograms of some selected plant extracts developed in EtOAc/MeOH/H_2O (60:20:5) solvent systems and sprayed with DPPH in methanol. Yellow zones indicate antioxidant activity.

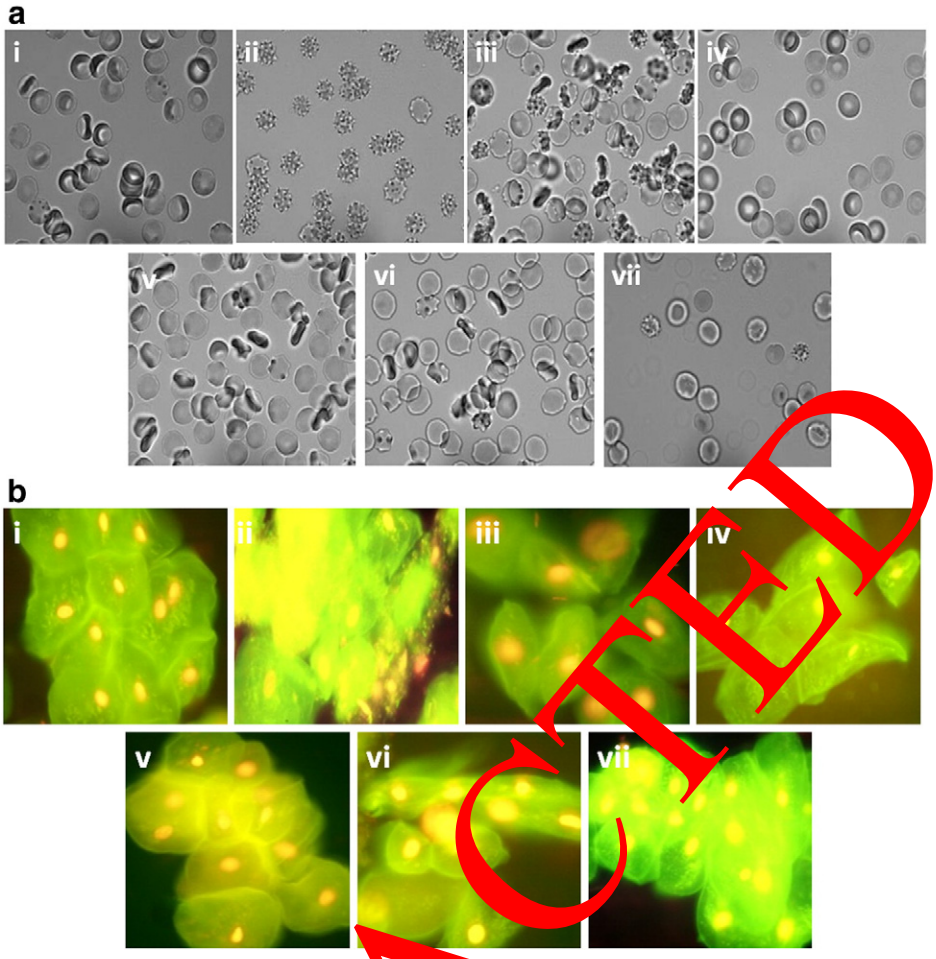

Fig. 2. (2a) Optical microscopic evaluation of the erythrocyte morphology. (i) Control—RBCs; (ii) RBCs plus oxidant; (iii) RBCs plus *O. tenuiflorum* extract; (iv) RBCs plus *L. aspera* extract; (v) RBCs plus *T. arjuna* extract; (vi) RBCs plus *G. glabra* extract; (vii) RBCs plus *N. arbortristis* extract at 50 µg/ml and oxidant after 1 h of incubation. (2b) Fluorescent microscopic evaluation of the buccal cell protection. (i) Control—buccal cells; (ii) buccal cells plus oxidant; (iii) buccal cells plus *O. tenuiflorum* extract; (iv) buccal cells plus *L. aspera* extract; (v) buccal cells plus *T. arjuna* extract; (vi) buccal cells plus *G. glabra* extract; (vii) buccal cells plus *N. arbortristis* extract at 50 µg/ml and oxidant after 1 h of incubation.

produce alterations in the erythrocyte membrane as manifested by a decreased cytoskeletal protein content and production of high molecular weight proteins which leads to abnormal erythrocyte shape (Battistelli et al., 2005). Hydrogen peroxide and ascorbate/Fe^{2+} induce an echinocytic type of shape alteration, characterized by protuberances over the cell membrane (Fig. 2a-ii), indicative of oxidative damage. From our results (Fig. 2a and b-iii–vii), it is evident that aqueous extracts of some of the selected medicinal plants *O. tenuiflorum*, *L. aspera*, *T. arjuna*, *G. glabra* and *N. arbortristis* were effective in bringing down the oxidative stress induced erythrocyte and buccal cell damage.

Quantitative antioxidant activity of herbal extracts was evaluated by DPPH radical scavenging and DNA protection assays (Meghashri et al., 2010). DPPH scavenging model system indicated free radical scavenging ability of the herbal extracts (Table 1). The aqueous extracts of *O. tenuiflorum*, *L. aspera*, *T. arjuna*, *G. glabra* and *N. arbortristis* showed highest free radical scavenging ability with IC_{50} of 13.74, 12.13, 19.04, 24.9 and 14.14 µg/ml respectively. These results showed the potential electron donating ability of the selected plant aqueous extracts. Also, DNA protective ability of herbal extracts was evaluated on lambda phage DNA oxidation (Fig. 3). The hydroxyl radical generated by Fenton's reagent caused DNA fragmentation with increase in its electrophoretic mobility (lane 2). This DNA fragmentation was recovered by treatment with herbal extracts (0.8 mg/16 µl). As evidenced by gel documentation analysis, higher protection (90%) was observed in *O. tenuiflorum*, *L. aspera* and *N. arbortristis* treated samples (lane 3–5); the other extracts did not show DNA protection on oxidation (data not shown).

Antifungal potential of the herbal extracts was evaluated against *R. stolonifer*. All the twenty extracts showed varying degree of activity. Appreciable inhibition was observed in *O. tenuiflorum*, *L. aspera*, *T. arjuna*, *G. glabra* and *N. arbortristis* extracts (Table 2). However, the genus *Ocimum* has frequently been used and the active constituents might be flavonoids (Jyoti et al., 2004). Highest fungal inhibition was observed in *L. aspera* whose studies have revealed the presence of phenolic compounds in its extract (Meghashri et al., 2010). The correlation co-efficient between *L. aspera* total phenolic content and cytoprotective activity, DPPH

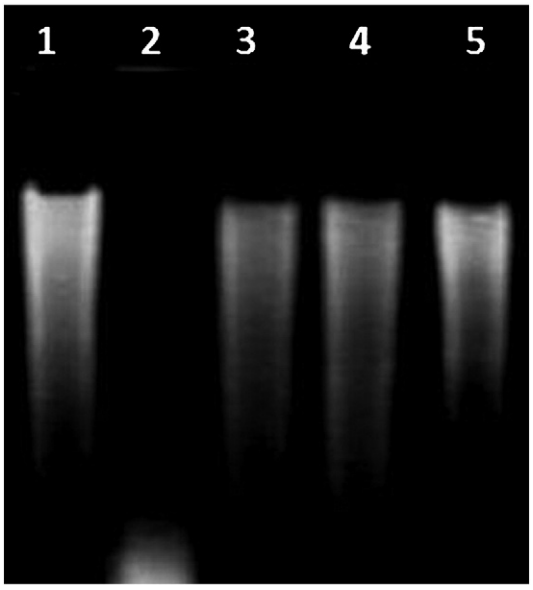

Fig. 3. DNA protection by *O. tenuiflorum, L. aspera* and *N. arbortristis* extracts (1 mg/ml). Lane 1—native DNA; lane 2—oxidized DNA; lane 3—*O. tenuiflorum* treated *DNA*; lane 4—*L. aspera* treated DNA; lane 5—*N. arbortristis* treated DNA.

radical scavenging activity and protection to DNA damage was r=0.9467, 0.9234 and 0.9781 respectively. This suggests the contribution of polyphenols in the plant extract bioactivity.

The present work demonstrated the antioxidant, cytoprotective and anti-*Rhizopus* activities of the selected plant extracts. The activities exhibited by some of the extracts could be the basis for their alleged health promoting potential. They could serve as rich sources of natural antioxidants or nutraceuticals with potential applications in reducing oxidative stress conditions.

Table 2
Antifungal activity of herbal extracts against *Rhizopus oryzae*.

Sample	Fungal growth inhibition (%)
1	68.07
2	38.26
3	13.85
4	15.01
5	24.84
6	14.56
7	26.91
8	20.43
9	10.2
10	70.06
11	48.12
12	23.91
13	65.2
14	61.9
15	18.79
16	12.6
17	5.72
18	26.55
19	32.16
20	26.2
Benzoic acid	65.9
BHA	60.40

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